Evaluation of Neurobehavioral Abnormalities and Immunotoxicity after Oral Imidacloprid Exposure in Domestic Chickens (*Gallus gallus domesticus*)

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Abstract

Neonicotinoid pesticides may have negative effects on non-target species at environmentally plausible exposure doses. The objective of the present study was to quantify neurobehavioral abnormalities and immunotoxicity due to oral imidacloprid exposure in birds. Domestic white leghorn chickens (*Gallus gallus domesticus*; n=120) were exposed to imidacloprid by gavage once daily for 7 consecutive days at 0, 0.03, 0.34, 3.42, 10.25, and 15.50 mg/kg. The severity and duration of neurobehavioral abnormalities were recorded, and immune function was assessed with 7 standard functional assays. Immunotoxicity was not detected. Temporary neurobehavioral abnormalities were observed in a dose-dependent manner, including generalized whole-body muscle tremors, ataxia, and depressed mentation ranging from mild depression to a complete lack of response to external stimulation. The effect dose value for the presence of any neurobehavioral abnormalities in 50% of the test group (ED_{50}) was 4.63 mg/kg/day. The ED_{50} for an adjusted score that included both the severity and duration of neurobehavioral abnormalities was 11.27 mg/kg/day. The no observed adverse effect level (NOAEL) and lowest observed effect level (LOEL) were 3.42 mg/kg/day, and the lowest observed adverse effect level (LOAEL) was 10.25 mg/kg/day. While immunotoxicity was not demonstrated in the present study, it cannot be ruled out. The observed neurobehavioral abnormalities were severe at the higher doses and may impair survival of free-living gallinaceous birds.

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INTRODUCTION

Neonicotinoids are a group of neuroactive insecticides which include acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid, and thiamethoxam. Neonicotinoids are currently the most widely used class of insecticides on the global market due to several factors including their relatively low vertebrate toxicity compared to other insecticides such as organophosphates, systemic chemical distribution within the plant, and efficacy against a wide range of insects at relatively low doses [1–7]. Neonicotinoids are highly versatile with multiple application methods for plants, including foliar sprays, granular soil applications, dipping of seedlings, mixing with irrigation water, and external seed coating [1–3]. Neonicotinoids are also used to eliminate household pests and for ectoparasite control in domestic animals [2]. Prophylactic seed coating is the most extensively used application method, with seed coating on corn constituting the largest single use of these compounds in North America [1,2]. When measured by geographic area, neonicotinoids are the most widely used class of agricultural insecticide in United States history, totaling 97.4 million hectares (240.7 million acres) in 2013 [1].

The widespread, often prophylactic, use of neonicotinoid pesticides and the associated environmental contamination has raised concerns about impacts on local ecosystems and non-target species [1,3,8–10]. Neonicotinoids are water soluble, frequently detected in natural watersheds, and can have prolonged half-lives in the soil depending on environmental conditions [3,4,8,9,11–16]. Birds seem to be particularly susceptible to adverse effects from neonicotinoids [3,17–19]. Birds might be exposed to neonicotinoids via multiple routes, including ingestion of treated seeds, ingestion of

contaminated invertebrates, direct contact with foliar spray, and contaminated water sources [17,19]. Reported sub-lethal effects of neonicotinoids in birds include neurobehavioral signs, immunotoxicity, weight loss and reduced reproductive success [3,17,19–29].

Oral neonicotinoid exposure in birds can cause substantial neurobehavioral abnormalities, including hypo-reactivity, ataxia, immobility, muscle tremors and respiratory distress [17,21,22,24]. White-crowned sparrows (*Zonotrichia leucophrys*) gavaged with imidacloprid at a dose equivalent to approximately 4 to 9 treated canola seeds were unable to correctly orient for migration and these effects persisted for at least 3 d past the last oral exposure [21]. The birds also developed respiratory distress, ataxia, lethargy, anorexia and weight loss [21]. South American eared doves (*Zenaida auriculata*) exposed to a single dose of oral imidacloprid exhibited dose-dependent neurobehavioral abnormalities including ataxia, depressed to non-responsive mentation, and spastic muscle contractions [22]. All of the aforementioned neurobehavioral signs could impair a wild bird's ability to survive and reproduce [17,21,22,24].

In addition to the severe neurobehavioral signs, immunotoxicity is a noteworthy sub-lethal effect because it has been linked with reduced survival probability and fitness in other systems [30–34]. Immune suppression associated with environmental toxicant exposure such as heavy metals, organochlorine pesticides and polychlorinated biphenyls has been documented in multiple avian species [34–39]. Nicotinic acetylcholine receptors are the target for neonicotinoid pesticides, and are present in several components of the immune system [40–42]. Exposure to neonicotinoids has resulted in immune suppression in bees, rats, mice and birds [28,29,43–51].

The objective of the present study was to facilitate ecological risk assessments by quantifying neurobehavioral abnormalities and immunotoxicity from oral imidacloprid exposure in birds in a dose-dependent manner. Domestic white leghorn chickens were used as a model for wild gallinaceous birds. The imidacloprid doses used in the present study were intended to be realistic potential field exposures for a medium-sized granivorous bird. The hypotheses of the present study were that oral imidacloprid exposure would cause neurobehavioral abnormalities in a dose-dependent manner, and detectable immune suppression.

MATERIALS AND METHODS

Study Design

Domestic chickens were obtained from a commercial poultry farm at 5 wk (n = 60) and 8 wk (n = 60) of age in 6 sequential groups (batches) over a 17 wk time frame. All chickens were raised in the same environment with the same diet prior to acquisition. Birds were vaccinated at the poultry farm with coarse spray vaccines as follows: Newcastle disease and infectious bronchitis at 2 wk of age, *Escherichia coli* and infectious bronchitis at 4 wk of age, and Newcastle disease, *Salmonella typhimurium*, and infectious bronchitis at 7 wk of age. At the research facility, chickens were identified with a numbered leg band and housed in groups of 10 to 15 separated by sex. The room lights were set for 8 h of light per day to decrease conspecific aggression among males. However, the majority of birds were housed in a room with some natural light, which was up to 15 h per day. When mild conspecific aggression was noted, the individual groupings were changed to resolve the conflict. Birds had ad libitum access to food and water and were given 7 to 8 d to acclimate prior to the study.

Birds were randomly assigned to 6 treatment groups containing 20 birds each, composed of 5 6-wk-old males, 5 6-wk-old females, 5 9-wk-old males, and 5 9-wk-old females. On intake exam, 15 birds had clinical signs consistent with mild respiratory tract infections (mildly depressed and mild respiratory crackles on air sac auscultation), and one bird had gastrointestinal signs (mildly depressed, decreased appetite and diarrhea). One bird received oral antibiotics (enrofloxacin 15 mg/kg by mouth once daily for 7 d) for the respiratory signs and returned to normal within 3 d of starting treatment. Respiratory signs self-resolved in the remaining 14 birds. The bird with gastrointestinal

signs was treated with gavage feeding and oral metronidazole (50 mg/kg by mouth twice daily for 5 d) and the clinical signs resolved within 3 d. Abnormal birds received subcutaneous fluids as indicated based on hydration status and appetite. All birds were deemed clinically healthy at the start of the study based on a physical exam and complete white blood cell count. All birds remained clinically healthy for the duration of the study. Imidacloprid exposures were calculated as percentages of the reported LD_{50} in domestic chickens (104.1 mg/kg) [20]. Nominal imidacloprid doses were 0.04% of the LD_{50} (0.04 mg/kg), 0.33% of the LD_{50} (0.34 mg/kg), 3.3% of the LD_{50} (3.44 mg/kg), 10% of the LD_{50} (10.41 mg/kg), and 15% of the LD_{50} (15.62 mg/kg), as well as a vehicle control group (0.00 mg/kg) (Table 1). Confirmed imidacloprid doses were 0.00, 0.03, 0.34, 3.42, 10.25, and 15.50 mg/kg (Table 1).

Birds were dosed by gavage once daily for 7 consecutive days (day 0-6) at approximately 24 h intervals (± 2 h; Figure 1). Clinical signs were monitored each day of oral exposure. Blood was collected for a complete blood count (CBC) and microbiocidal assay immediately prior to oral exposure on days 0, 3, 7, 14 and 21. The PHA test was performed on days 7 to 8. Birds were exposed to *Mycobacterium tuberculosis* antigen on day 1 for the delayed type hypersensitivity (DTH) test, and a tuberculin skin test was performed on days 14 to 15. Birds were exposed to SRBC antigens on day 1, and blood was collected for agglutination and hemolysis titers on day 7. Birds were humanely euthanized for a complete gross necropsy and tissue collection on day 21. The research protocol was approved by the University of Minnesota Institutional Animal Care and Use Committee (protocol # 1610-34271A).

Oral exposure

A chemical standard of imidacloprid powder (Cat # N-1226-100 mg, 99.4% purity, ChemService Inc.) was dissolved in a vehicle solution of corn oil and 10% ethanol. The highest concentration solution was prepared by dissolving neat material in ethanol, then mixing thoroughly with corn oil using sonication. The lower concentration solutions were prepared by diluting the highest concentration solution with corn oil. The solution concentrations were confirmed using high performance liquid chromatography (HPLC) tandem mass spectrometry (MS) on an Agilent 1260 HPLC system interfaced with a 3200 QTrap triple quadrupole/linear ion trap MS (AB Sciex; Toronto, Canada) and equipped with a ZORBAX Extended-C18 column (100 × 2.1 mm, 3.5 μm, 80 Å, Agilent Technologies) (Table 1). The MS was equipped with a TurboIonSpray® electrospray ionization (ESI) probe operated in the multiple reaction monitoring (MRM) mode. The mobile phase consisted of methanol (A) and water (B), both spiked with 0.1% formic acid (v/v). The mobile phase flow rate was 200 μL/min and the following gradient was employed: 10% B ramped to 70% B in 11 min (linear) and then ramped to 80% B in 6 min (linear), followed by a linear increase to 90% B in 2 min (held for 1 min) and then a change to 10% B in 1 min (held for 8 min). Five concentrations of imidacloprid suspension were used so each bird received approximately the same amount of vehicle (2 mL/kg). The 0.00 mg/kg vehicle control group was exposed to an equivalent volume of vehicle solution. The imidacloprid solutions were protected from light to avoid photolysis and mixed thoroughly prior to each use. Birds were weighed to the nearest gram immediately prior to each oral exposure and the appropriate solution volume was calculated based on the daily weight. The assigned imidacloprid exposure was mixed

with a grain-based feeding formula at a total volume of 1.5% of the daily body weight. Birds were manually restrained, a lubricated feeding tube was passed into the ventriculus, the exposure solution and feeding formula were administered, then the tube was flushed with 3 to 6 mL of warm water to ensure the entire volume was administered. No regurgitation was noted. The size of the crop was scored as follows by 2 trained researchers as a proxy for how full the gastrointestinal tract was prior to gavage: 0 empty, 1 mildly full, 2 moderately full, 3 very full.

Neurobehavioral abnormalities

Neurobehavioral signs were scored each day of oral exposure by 2 trained researchers based on set criteria derived from the modified Glasgow coma scale used in veterinary medicine (Table 2) [52]. Both observers saw all birds in each exposure group. The housing and research set up precluded the observers from being blinded. However, treatment group was not confirmed prior to assigning a clinical severity score, and researchers adhered to the defining characteristics of each severity score. Birds were monitored for clinical signs every 5 to 10 min immediately after gavage, then hourly once the most severe neurobehavioral signs were reached. Birds received one clinical severity score on each exposure day indicating the most severe neurobehavioral signs displayed that day. The time from gavage to onset of any neurobehavioral signs, and the approximate duration of neurobehavioral signs from the time of onset to complete resolution of signs, were recorded in min.

Immune function assays

Multiple immune function assays were performed in order to evaluate components of both the innate and adaptive immune system [33,34,53]. Assays were chosen from both tier I and II of the National Toxicology Program (NTP) guidelines for immunotoxicity [54,55]. These assays are an accurate predictor of immunotoxicity in rodents and birds at sublethal toxicant exposures, especially when used in combination [34,54–57].

Complete blood counts. Blood was sterilely collected into a heparinized syringe within 3 min of manual restraint, prior to all other procedures, in order to minimize the effects of stress on the results. Packed cell volume (PCV) and buffy coat (BC) percentages were measured using heparinized hematocrit tubes. Total solids (TS) were measured with a calibrated refractometer. Estimated total white blood cell (WBC) counts were performed manually with a blood smear [58,59]. All blood smears were read by the same experienced technician in a random fashion without knowledge of the exposure group. Chronic stress can result in immunosuppression in birds, and is demonstrated by an increase in the H/L ratio and WBC count [60–64].

Microbiocidal assay. The microbiocidal assay measures the ability of the innate immune system to kill a known quantity of bacteria or yeast [65,66]. Different microbe species initiate variable responses from the immune system; therefore, by evaluating the killing ability of plasma against 3 different classes of microbes, multiple mechanisms were assessed [65–68]. Blood was sterilely collected into a heparinized syringe, plasma was separated into aliquots and stored at -80°C until analysis. Lyophilized pellets of *Escherichia coli* (ATCC #8739), *Staphylococcus aureus* (ATCC #6538) and *Candida*

albicans (ATCC #10231) were reconstituted according to the manufacturer instructions (Epower™ Microorganisms, Microbiologics®). The methodology was as published in French and Neuman-Lee, 2012 with the following modifications [66,68]. Thawed plasma was diluted 1:3 in phosphate buffered saline for *S. aureus* and *C. albicans*. Plasma was diluted 1:5 in CO_2 independent media for *E. coli*. Tryptic soy broth was added to all wells at a 1:8 dilution for *S. aureus* and *C. albicans*, and a 1:6 dilution for *E. coli*. The microbiocidal ability of plasma is reported as the percentage of microorganisms killed by the plasma, calculated as follows: ((1 – sample mean absorbance/positive control mean absorbance)*100).

The *S. aureus* and *C. albicans* analyses were performed 6 to 9 wk after sample collection. *S. aureus* and *C. albicans* results are not available from the first batch of birds (n=5 from the 0.03, 0.34, 3.42, and 10.25 mg/kg groups, and n=4 from the 0.00 mg/kg group) because the plasma samples were degraded from freezer storage when the assay methodology was validated, and the results were no longer comparable to the other batches. The *E. coli* analysis was performed 12 to 13 mo after sample collection and was staged so that all samples were stored for the same duration of time prior to analysis to correct for possible sample degradation.

Sheep red blood cell hemagglutination and hemolysis. The SRBC hemagglutination and hemolysis assay evaluates the ability of B lymphocytes to generate a primary, antigen-specific antibody response after a single exposure to a novel antigen, and the ability of natural and acquired antibodies to initiate SRBC lysis via the complement cascade [33,64,69,70]. Blood was sterilely collected into a heparinized syringe; plasma was separated into aliquots and stored at -80°C until analysis. The same

vial of 50% whole sheep blood and Alsever solution (Colorado Serum Company) that was used for injection into an individual chicken was used for the bench top assay for that bird to ensure that the antigen profile of the SRBCs was identical. The SRBCs were processed as published in Grasman, 2010 [33]. The hemagglutination and hemolysis methodology was as published in Matson *et al.*, 2005 aside from the following modifications [69]. Birds were injected with 0.1 mL of a 20% SRBC solution into the left pectoral muscle. Thawed, un-heated plasma was serially diluted 1:2, resulting in dilutions from 1:2 to 1:2,048. Samples were run in duplicate on separate plates. A 1% SRBC solution was added to all wells at a 1:3 dilution. The final agglutination titer was expressed as the $\log_2$ of the mean reciprocal titer. All plates were scored by one researcher.

The hemolysis results are expressed as the plasma dilution required to produce 50% lysis of the SRBCs (CH_{50}) [70]. Saline was used as a negative SRBC lysis control, and distilled water was used as a positive lysis control. The mean absorbance of the positive control wells equated to 100% SRBC lysis. The background absorbance of the plasma was read at 405 nm prior to adding SRBCs to the plate and was subtracted from the final absorbance. The final absorbance of the supernatant was measured after the last incubation period to evaluate SRBC lysis [69]. The percent lysis of each well was calculated using the following formula: ((percent lysis = sample absorbance/mean absorbance of the positive controls)*100). The CH_{50} for each sample was calculated as in Costabile, 2010, and the results were averaged between the duplicate plates [70].

Phytohemagglutinin-A. The PHA response is primarily an adaptive immune response orchestrated by T lymphocytes, however the innate immune system contributes

to the inflammation measured as the skin swelling [33,71,72]. The procedure for the PHA test was based on published avian protocols [33,71]. Feathers were plucked from an approximately 1 cm diameter area on the left patagium 48 h prior to the PHA assay to allow any inflammation associated with feather removal to resolve. The patagial thickness in the plucked area was measured to the nearest 0.01 mm using a digital micrometer. Three measurements were taken with approximately 50% location overlap and averaged. PHA (lectin from the red kidney bean (*Phaseolus vulgaris*)), 0.1 mg (0.1 mL, 5 mg/mL), was injected subcutaneously and the injection site was marked with a permanent marker. The skin thickness was measured approximately 24 h (± 3-4 h) after the PHA injection via the same method. All measurements were taken by the same researcher. The immune response is characterized by the amount of swelling after the PHA injection, presented as the post-PHA injection measurement subtracted from the pre-PHA injection measurement in mm.

Delayed type hypersensitivity. The DTH test evaluates the ability to produce antigen specific memory T cells in response to *Mycobacterium tuberculosis* in Freund's complete adjuvant, a slow release medium that enhances the Th1 CD4+ T lymphocyte response [64,73,74]. Two weeks after the initial injection, birds were injected with tuberculin purified protein derivative (PPD), which stimulates a complex cascade of immune responses involving CD8+ T cells, CD4+ T cells, and cytokines, resulting in a type IV hypersensitivity reaction and a measurable skin swelling [64,73]. The DTH test was modified from methods used in domestic chickens [64,73]. Chickens were injected subcutaneously over the right pectoral muscle with 0.5 mg of killed *Mycobacterium tuberculosis* (H37Ra) in Freund's complete adjuvant. The baseline thickness of the

interdigital skin web between digits 3 and 4 was measured to the nearest 0.01 mm on both feet using a digital micrometer. Three measurements were taken with at least 50% location overlap and averaged. An intradermal injection of 0.1 mL of PPD diluted 1:1 with sterile saline was given in the right interdigital skin web. The same procedure was repeated on the left foot with 0.2 mL of sterile saline as a negative control. The thickness of both interdigital skin webs was measured 24 h (± 3-4 h) later via the same method by the same researcher. The results are expressed as follows: ((mean post PPD injection measurement/mean pre-PPD injection measurement) – (mean post saline injection measurement/mean pre-saline injection measurement)).

Necropsy. Birds were humanely euthanized with an intravenous injection of euthanasia solution (1 mL, 390 mg/mL pentobarbital sodium, 50 mg/mL phenytoin). Within 5 h of euthanasia, a complete gross post-mortem examination and tissue collection was performed. Sex was confirmed and any gross abnormalities were noted. Organ weights were obtained for the spleen, thymus, bursa of Fabricius, liver, kidney and brain, and were expressed as percentages of bird body weight at the time of euthanasia.

Statistical methods

The mean clinical severity score, time (min) until onset of neurobehavioral signs, and duration of neurobehavioral signs (min) were calculated for each individual bird over the duration of the exposure period (day 0-6). The mean value for each individual was used to calculate the treatment group mean. All birds were included in the treatment group mean clinical severity score and duration of neurobehavioral signs calculations, including birds with clinical severity scores of 0 and duration values of 0 min. Only birds

that developed neurobehavioral signs were included in the treatment group mean min for onset of neurobehavioral signs. Mean clinical severity scores, mean time until onset, and mean duration of neurobehavioral signs were compared between treatment groups with a Kruskal-Wallis test. The hypotheses were that the clinical severity score and duration of clinical signs would increase significantly with increasing dose of imidacloprid, and that the time until onset would decrease with increasing dose of imidacloprid. T-tests were used to compare the clinical severity scores and duration of neurobehavioral signs between sexes within the 3.42, 10.25 and 15.50 mg/kg treatment groups. The hypothesis was no difference existed in the clinical severity score and duration of neurobehavioral signs between sexes. The correlation between the individual bird mean clinical severity score and individual mean duration of neurobehavioral signs was analyzed with linear regression. The hypothesis was the duration of neurobehavioral signs would increase in a linear fashion with increasing clinical severity score. To evaluate change in individual clinical severity scores and duration of neurobehavioral signs over time, a linear regression line was fitted for each bird in the 3.42, 10.25 and 15.50 mg/kg treatment groups. The slope of the linear regression line was used as a representation of how an individual changed over time. A Wilcoxon signed-rank test was performed within each treatment group to test if the slope values were significantly different from zero. A Kruskal-Wallis test was performed to compare the mean slope between treatment groups. The hypothesis was the slope of the clinical severity scores and duration of neurobehavioral signs within individual birds would not differ from zero, and there would be no difference in slopes between treatment groups.

The no observed effect level (NOEL), no observed adverse effect level (NOAEL), the lowest observed effect level (LOEL) and the lowest observed adverse effect level (LOAEL) were based on statistically significant differences between treatment groups using both the daily and the individual mean clinical severity scores. The effect dose values where 50% (ED_{50}) and 10% (ED_{10}) of the population was affected were determined by fitting the observed neurobehavioral signs with a 4 parameter log-logistic dose response model using the drc package in R 3.4.3 open-source environment [75,76]. The clinical severity scores were adjusted via 2 methods for this analysis: the presence of any neurobehavioral signs where zero indicated no neurobehavioral signs and one indicated any type of neurobehavioral sign, and an adjusted score, calculated as the logarithm of the duration of neurobehavioral signs multiplied by the square root of the observed clinical severity score.

Weekly weight gain was compared between treatment groups via an analysis of covariance (ANCOVA) for each time interval. Sex was included as a covariate because males had a higher growth rate than females. The hypothesis was that weekly weight gain would decrease in a dose-dependent manner associated with oral imidacloprid exposure. Weight comparisons between treatment groups were limited to within each weekly time interval; no comparisons were made between time intervals. Mean crop size was compared between treatment groups via a one-way analysis of variance (ANOVA) on each exposure day. The hypothesis was that mean crop size would not differ between treatment groups throughout the exposure period. Spearman rank-order correlation was used to assess if there was a monotonic relationship between crop size and clinical

severity score. The hypothesis was that crop size would not be correlated with clinical severity score.

The immune function assays with one data point were analyzed via an ANOVA, followed by post-hoc pairwise t-tests with Bonferroni correction. When normality assumptions were not met, a Kruskal-Wallis one-way analysis of variance was performed, followed by Dunn Kruskal-Wallis multiple comparisons with Bonferroni adjustment. Continuous data with repeated measurements within an individual bird were analyzed with a linear-mixed model with batch and chicken identification as random effects. Batch was included in all models to reflect the block study design. Sex and age were evaluated as potential confounding variables in the linear-mixed model and were included in models where they had a statistically significant and biologically relevant effect on the results. Confounding variables in the linear-mixed models were assessed using an ANOVA. The hypotheses for the immune function assays were that immune function would significantly decrease in response to oral imidacloprid exposure in a dose-dependent manner. A power analysis was performed for the PHA, DTH, SRBC hemagglutination and SRBC hemolysis assays and all had a statistical power of 80% or greater with a significance level of 0.05. An alpha level of 0.05 was used for significance.

RESULTS

Neurobehavioral abnormalities

No neurobehavioral abnormalities were noted prior to the first imidacloprid exposure on day 0. Neurobehavioral abnormalities were only observed after oral imidacloprid exposure, and all birds returned to normal prior to the subsequent dose. No neurobehavioral abnormalities were noted in the 0.00 or 0.03 mg/kg birds at any point during the exposure period. Only one bird developed neurobehavioral signs in the 0.34 mg/kg group, exhibiting mild neurobehavioral signs on day 1 and 2, with a mean ± standard deviation of 65 ± 49.5 min until onset and a mean duration of 45 ± 21.2 min. The percent of birds that developed any neurobehavioral signs on each exposure day increased with increasing dose of imidacloprid (Figure 2). The percent of birds in each treatment group that developed any neurobehavioral signs at any point during the study, calculated as the number of days where any neurobehavioral signs were observed divided by the total number of days of imidacloprid exposure, was 1.4% of 0.34 mg/kg, 30% of 3.42 mg/kg, 92.9% of 10.25 mg/kg, and 98.6% of 15.50 mg/kg birds (Pearson chi-squared test; $P < 0.001$). All of the 15.50 mg/kg birds developed neurobehavioral signs on every day of exposure except for days 3 and 6, when a single bird remained clinically normal. A different female bird remained normal each day.

Birds exhibited a gradient of neurobehavioral signs each day of exposure. Neurobehavioral signs started with mild abnormalities, increased in severity to the most severe signs exhibited that day, then gradually decreased in severity until returning to normal. Affected animals were unable to ambulate normally due to ataxia and generalized muscle tremors, had decreased responsiveness to external stimulation, and in

some cases were completely nonresponsive to their surroundings. These neurobehavioral abnormalities persisted for over 3 h in the most severe cases. The mean time until onset of clinical signs ± standard deviation was 26.0 ± 12.5 mins for the 3.42 mg/kg group, 19.2 ± 10.9 mins for the 10.25 mg/kg group, and 12.6 ± 3.7 mins for the 15.50 mg/kg group. Despite the inverse relationship between time until onset and dose, a statistically significant difference in the time until onset of neurobehavioral signs was not detected between the 3.42, 10.25, and 15.50 mg/kg treatment groups (Kruskal-Wallis chi-squared = 2.20; $p = 0.14$). The most rapid onset of neurobehavioral signs was noted 3 min post-gavage in a female 10.25 mg/kg and a male 3.42 mg/kg bird.

The mean clinical severity score for the 10.25 and 15.50 mg/kg treatment groups was higher than all other groups ($p \leq 0.008$; Figure 3). The mean clinical severity score for the 3.42 mg/kg group was marginally higher than the 0.00 mg/kg group ($p = 0.052$). Males within the 10.25 and 15.50 mg/kg treatment groups had higher mean clinical severity scores compared to females within the same group, and the disparity between sexes increased in the higher exposure group ($p \leq 0.03$). The mean slope of the clinical severity scores declined over the exposure period within the 15.50 mg/kg group, and this relationship was driven by females (Table 3). The only statistically significant difference in the clinical severity slope when the sexes were combined was between the 10.25 and 15.50 mg/kg groups ($p = 0.02$). No statistically significant differences were detected in the mean clinical severity slope between treatment groups in males ($p \geq 0.76$). In females, the clinical severity slope in the 15.50 mg/kg group was significantly lower than the 10.25 mg/kg group ($p = 0.04$).

The mean duration of neurobehavioral signs increased in a dose-dependent manner, with the duration of neurobehavioral signs in the 10.25 and 15.50 mg/kg groups being significantly longer than all other groups ($p \leq 0.002$; Figure 4). The 10.25 and 15.50 mg/kg treatment groups did not differ from each other ($p = 1.0$), nor did the 3.42 mg/kg treatment group differ from the 0.00, 0.03, or 0.34 mg/kg groups ($p \geq 0.089$). The mean duration of neurobehavioral signs was longer in males than females within the 10.25 mg/kg group only ($p = 0.001$). The mean slope of the duration of neurobehavioral signs declined significantly within the 15.50 mg/kg treatment group, and this change was driven by the female birds (Table 4). When the sexes were combined, the mean slope was slightly lower in the 15.50 mg/kg group compared to the 3.42 mg/kg group ($p = 0.052$), but none of the comparisons between treatment groups were statistically significant. When separated by sex, no significant differences in slope were detected between treatment groups in males. In females, the mean slope in the 15.50 mg/kg group was significantly lower than the 3.42 and 10.25 mg/kg groups ($p \leq 0.018$). A linear regression confirmed a correlation between a higher individual mean clinical severity score and a longer individual mean duration of neurobehavioral signs (Figure 5).

Severity of neurobehavioral signs within an individual bird varied from one exposure day to the next in the 3.42, 10.25, and 15.50 mg/kg groups. For example, one male 15.50 mg/kg bird had a clinical severity score of 2 on day 1, a score of 4 on day 2 and 3, and then returned to a score of 2 on day 4. No clear pattern was evident for the change in clinical severity scores within an individual bird between exposure days, and it did not appear to be related to exposure dose. The variability may have been related to

crop size, but this was not the only explanatory factor (Spearman rank correlation crop size and clinical severity score, $r_2 = 0.22$, $p < 0.001$).

Weight gain and crop size. The mean weekly weight gain from day 0 to 7 in the 15.50 mg/kg group was nearly 50% lower than the other groups ($p < 0.001$; Figure 6). After the cessation of imidacloprid exposure, the mean weekly weight gain from days 7 to 14 in the 15.50 mg/kg group was approximately 25% greater than the other groups ($p \leq 0.007$). No other differences in weekly weight gain were detected between treatment groups ($p \geq 0.07$). No significant differences in the mean total weight gain from day 0 to 21 were detected between treatment groups ($p \geq 0.41$).

The crop size in the 15.50 mg/kg group was significantly larger on days 4 and 5 (mean score ± standard deviation; 2.2 ± 1.0 and 2.1 ± 0.9 respectively) compared to the 0.00, 0.03, 0.34, and 3.42 mg/kg groups (mean score 0.9 ± 0.8-1.3 ± 0.6; $p \leq 0.024$). The crop size in the 15.50 mg/kg group was significantly larger on day 1 (2.1 ± 0.8) compared to the 0.00, 3.42, and 10.25 mg/kg groups (1.0 ± 0.7-1.3 ± 0.7; $p \leq 0.019$). On day 6, the crop in the 10.25 mg/kg group was significantly larger (1.8 ± 0.8) compared to the 0.00 mg/kg group (0.9 ± 0.7; $p = 0.017$).

Effect levels

The observed effect levels were determined using the individual mean clinical severity score and the daily clinical severity scores. Using the individual mean clinical severity score for each treatment group, the NOEL was 0.34 mg/kg/day, the NOAEL and LOEL were 3.42 mg/kg/day, and the LOAEL was 10.25 mg/kg/day. The observed effect levels using the daily clinical severity scores for each exposure day were the same as

those calculated using the individual mean clinical severity score. The dose-response curves for the presence of any neurobehavioral signs and the adjusted scores increased rapidly beginning at imidacloprid doses of 3.42 mg/kg/day (Figure 7). The ED_{10} for the presence of any neurobehavioral signs was 2.19 ± 0.51 mg/kg/day, and the ED_{50} was 4.62 ± 0.98 mg/g/day. The ED_{10} for the adjusted score was 2.54 ± 0.88 mg/kg/day, and the ED_{50} was 11.24 ± 9.33 mg/kg/day.

Immune function assays

Complete blood counts. Batch and sex did not influence the CBC parameters. The linear-mixed model for PCV and TS included age. Mean PCV did not differ between treatment groups at any time point (Table 5). On day 0, the mean TS in the 15.50 mg/kg group was significantly lower than the 0.03 and 10.25 mg/kg treatment groups ($p \leq 0.03$). No differences were detected in TS on days 3, 7, 14 and 21 ($p > 0.05$). The mean TS within all treatment groups increased over time ($p \leq 0.03$) and was reflected by the significant treatment group and time interaction ($p = 0.009$).

Final linear-mixed models for the total WBC count and the H/L ratio did not include age or sex. The total WBC count increased significantly from day 0 to 21 in all treatment groups ($p \leq 0.02$; Table 5) except the 0 mg/kg group, where the total WBC count increased from day 0 to 21 but it was not statistically significant. The highest mean total WBC count was recorded on day 21 for all groups except the 3.42 mg/kg group, which had the highest mean total WBC count on day 14. The H/L ratio increased from day 0 to 21 within all treatment groups ($p \leq 0.01$; Table 5), except the 0 mg/kg and 15.50 mg/kg groups. The increase in H/L ratio started after day 7 in all treatment groups. The

mean total WBC count and H/L ratio did not differ between treatment groups on any of the sampling days ($p > 0.05$).

Microbiocidal assay. The linear-mixed model for *S. aureus*, *C. albicans* and *E. coli* did not include sex or age. The mean percent killing of *S. aureus*, *E. coli* and *C. albicans* did not differ between treatment groups on any of the sampling dates (Figure 8). The percent killing of *S. aureus*, *E. coli* and *C. albicans* did change over time within treatment groups (Figure 8). The percent killing of *S. aureus* gradually increased from day 0 to 21 in the 3.42 mg/kg group ($p \leq 0.03$) and from day 3 to 21 in the 10.25 mg/kg group ($p \leq 0.03$). The percent killing of *E. coli* decreased by 17% from day 0 to 14 in the 15.50 mg/kg treatment group ($p = 0.03$). The percent killing of *C. albicans* decreased from day 0 to 7 in the 0.03, 10.25 and 15.50 mg/kg groups ($p \leq 0.02$) and then increased towards baseline levels on day 14.

Other immune function assays. Mean SRBC agglutination titers ($p = 0.13$), CH_{50} hemolysis titers ($p = 0.60$), PHA response ($p = 0.84$), and DTH response ($p = 0.78$) did not differ between treatment groups (Table 6). The exact time from injection to skin measurement did not affect the results for the PHA or DTH assay.

Necropsy. Organ weights did not differ between treatment groups ($p \geq 0.16$; Table 6), and brain weight was consistently 2 to 3 g. All birds developed multifocal mass lesions overlying the right pectoral muscle. The lesions contained caseous debris encapsulated by fibrous tissue with varying degrees of associated vascular formation. Samples were aseptically collected from 3 individuals and were negative for bacterial growth via aerobic and anaerobic culture. Six samples were analyzed via histopathology, one bird was represented from each treatment group. The mass lesions were diagnosed as

chronic, multifocal granulomatous cellulitis, consistent with adjuvant induced granulomas [77]. No infectious organisms were observed on histology. The severity of abscess formation was subjectively scored by one researcher as mild, moderate or severe, and was not correlated with treatment group, batch, sex or age ($p > 0.05$). Sex determination was confirmed as correct in all birds. No other abnormalities were noted on gross necropsy.

DISCUSSION

In the present study, chickens developed dose-dependent, temporary neurobehavioral abnormalities after oral imidacloprid exposure. While the neurobehavioral signs were comparable to those observed in passerine and gallinaceous species in field and laboratory settings [17,19,21,22,24,78–81], the effects were precisely documented in a dose-response study which allowed for determination of effect dose levels. The nervous system appeared to be a more sensitive indicator of sublethal effects than the immune system, as no detectable immunotoxicity secondary to oral imidacloprid exposure occurred at the doses and duration of exposure utilized in the present study.

Neurobehavioral abnormalities

Chickens exposed to field-realistic doses of imidacloprid developed neurobehavioral signs ranging from mild sedation in the mid-dose group to complete immobility and lack of response to external stimulation in the most severely affected birds in the high-dose group. These abnormalities would likely compromise the ability of a wild bird to evade predation and injury, as well as to perform normal biological functions such as foraging for food. Similar hypotheses have been proposed regarding impaired survival in birds exposed to other neurotoxicants such as lead and organophosphates, but sublethal effects are challenging to document in field settings [82–84].

The neurobehavioral signs observed in the present study were most likely caused by reversible binding of imidacloprid and/or imidacloprid metabolites to avian nAChRs in the nervous system and at neuromuscular junctions, inhibiting normal

neurotransmission. Neonicotinoids eliminate insect pests by binding and overstimulating nAChRs, resulting in paralysis, cell exhaustion and death [1,3,5,6,85]. Despite the intended specificity for invertebrate nAChRs, imidacloprid exhibited similar excitatory effects as nicotine on nAChRs in rat brain tissue, and acted as an agonist at human nAChRs [86,87]. Mammals can metabolize imidacloprid to a desnitro metabolite that can bind to mammalian nicotinic receptors [1,6,88–90]. Additionally, Japanese quail (*Coturnix japonica*) exposed to imidacloprid produced 5-hydroxy and olefin metabolites, 2 of the same metabolites as mammals [91]. Like mammals, birds may be able to metabolize imidacloprid to a form that can more readily bind to avian receptors, resulting in the observed neurobehavioral signs.

In the present study, birds that received larger doses of imidacloprid had significantly higher clinical severity scores, which suggests that larger doses resulted in more widespread nAChR stimulation, causing more severe neurologic effects. Additionally, a direct positive correlation existed between a higher clinical severity score and a longer duration of neurobehavioral signs, with abnormalities lasting up to 5.5 h with a clinical severity score of 4 in the high-dose group. The increasing severity and longer duration with increasing dose follows a typical dose-response relationship, with higher doses resulting in more widespread receptor stimulation and a longer time to metabolism, excretion and resolution [92]. A positive relationship was noted with more rapid onset of neurobehavioral signs at higher doses; however, this relationship was not statistically significant. With neurobehavioral signs developing in under 5 min in the most rapid cases, wild birds could quickly succumb to the effects of imidacloprid and may be unable to find cover before becoming incapacitated.

Neurobehavioral signs were temporary and cumulative toxicity from imidacloprid exposure was not observed in the present study. This is supported by the complete resolution of neurobehavioral signs between oral exposures, and the absence of increasing clinical severity scores over time and the static effect levels calculated on each exposure day. These findings suggest that chickens absorb and excrete imidacloprid relatively quickly, and that each exposure day during this 7 d exposure study was an independent time point with respect to neurobehavioral abnormalities rather than one, cumulative subacute exposure. This is consistent with the rapid metabolism observed in Japanese quail, where imidacloprid was cleared from the blood, brain, muscle, liver and kidney within 24 h after a single exposure to wheat seeds treated with 3 and 9% of the LD_{50} of imidacloprid [91].

Possible causes of the variability in clinical severity scores over time within individual birds include differing amounts of food present in the ventriculus at the time of gavage, differences in chemical absorption, and inconsistent concentration of the imidacloprid solutions. Because birds had free access to food at all times, the amount of food in the ventriculus at the time of oral exposure was variable between individual birds and exposure days. The presence of food in the ventriculus can reduce the pH, which can change chemical absorption [92]. Additionally, neonicotinoids are primarily water soluble and may be better absorbed on an empty stomach, whereas lipid-soluble substances are better absorbed with food [1,92]. Inconsistent concentration of the imidacloprid solutions is considered unlikely as solutions were thoroughly mixed prior to each dose.

In the highest exposure group, the clinical severity score and duration of neurobehavioral signs declined significantly over time. However, the small value of the declining slopes may not translate to a biologically relevant effect on survival probability. Declining clinical severity score and duration over time in the high exposure group might suggest up-regulation of enzymes associated with chemical metabolism, such as cytochrome enzymes, resulting in more rapid chemical metabolism, decreased severity of neurobehavioral signs, and a shorter duration [93,94]. However, enzyme up-regulation in response to neonicotinoids was not found in male Japanese quail after one or 10 doses of oral imidacloprid [91]. The underlying mechanism for higher mean clinical severity scores in males and the declining clinical severity scores in females is unknown. Sex hormones could play a role, as male rats sustained more genotoxic effects from imidacloprid than females, which was hypothesized to be caused by effects of sex hormones on imidacloprid metabolism [95].

The decreased weight gain during the exposure period in the high-dose group was likely caused by the severe neurobehavioral signs the birds experienced, resulting in decreased food consumption. The growing 6 to 13 wk old birds used in the present study have higher metabolic needs than adult birds, and therefore have a higher daily caloric requirement [96,97]. Other studies have also documented decreased weight gain in growing birds associated with neonicotinoid exposure, as well as weight loss in adult birds [17,19,28,98]. The increased weight gain in the high-dose group the week after the cessation of imidacloprid exposure may have been due to compensatory food consumption as a result of reduced weight gain the week prior. The increased crop size in the high-dose group may be a reflection of increased food consumption prior to the daily

oral exposure relative to the other birds that were neurologically normal or had a shorter duration of neurologic signs and could distribute their daily caloric intake throughout the day. Decreased weight gain or weight loss secondary to imidacloprid exposure could have important survival and reproductive impacts for birds, for example neurobehavioral deficits may impair food procurement during the high energetic demands of egg-laying [99–101].

Effect levels

The effect levels determined from the neurobehavioral signs observed in the present study occurred at field-realistic doses of imidacloprid. These values provide important information about sublethal neurobehavioral effects that may impair survival of wild gallinaceous birds. Labels on widely used commercial imidacloprid products specify that soybean seeds can contain 0.16 mg/seed, and wheat seeds can contain 0.033 mg/seed [19,102]. For illustrative purposes, these seed application rates can be translated into the estimated number of treated seeds a bird would need to ingest to reach various endpoints, such as the observed effect levels calculated in the present study. However, variables such as application rate, environmental degradation, and concurrent exposure to other chemicals present on treated seed will alter the anticipated numbers of seeds that must be ingested to result in neurobehavioral signs [3,8,11,17,19,102]. Based on the average consumption rate of 126 untreated wheat seeds per feeding bout in an approximately 1 kg ring-necked pheasant (*Phasianus colchicus*; = 4.2 mg/kg imidacloprid), and 55 untreated wheat seeds per feeding bout for an approximately 0.5 kg red-legged partridge (*Alectoris rufa*; = 3.6 mg/kg imidacloprid) [103], and conservatively assuming that consumption

rates between treated and untreated seeds are similar, these gallinaceous birds could consume enough imidacloprid treated seeds to reach the effect levels observed in the present study in a single feeding (presence of any neurobehavioral sign ED_{10} = 2.19 ± 0.51 mg/kg/day, ED_{50} 4.62 ± 0.98 mg/g/day; adjusted score ED_{10} = 2.54 ± 0.88 mg/kg/day; NOEL = 0.34 mg/kg/day; NOAEL and LOEL = 3.42 mg/kg/day).

Exposure to neonicotinoids via ingestion of treated seed has been debated based on the removal of the outer seed husks where the majority of pesticide is located by some birds, environmental degradation of the pesticide prior to seed ingestion, as well as learned avoidance of treated seed after experiencing ill effects post-ingestion [17,19,103–106]. Nevertheless, cases of toxicity in wild birds have been documented due to ingestion of neonicotinoid treated seed, including neurologic signs, internal trauma and death in Cape spurfowl (*Pternistis capensis*), grey partridges (*Perdix perdix*), pigeons (*Columba sp.*), and red-winged blackbirds (*Agelaius phoeniceus*) [17,19,24,78–81]. The increased crop size in the high-dose group indicates that food aversion associated with imidacloprid exposure did not occur in the present study, however birds were exposed via gavage not voluntary ingestion of treated seed, and this cannot be separated from a decrease in gastrointestinal motility. Delayed crop emptying has been associated with systemic disease and toxicants such as lead and organophosphates in birds [107–109], and nicotinic acetylcholine receptors play a role in gastrointestinal motility in humans [110]. Therefore, inhibition of nAChRs in birds may result in impaired gastrointestinal motility, which may be the underlying cause of the increased crop size observed in the high-dose birds.

Immune function

Despite the reported immunotoxicity associated with neonicotinoids in birds[28,29,43,51]**,** domestic chickens exposed to oral imidacloprid in the present study did not exhibit detectable immune suppression or stimulation. Possible causes of the increase in the total WBC count and H/L ratio over time in all groups include handling stress, a generalized inflammatory reaction associated with the sterile abscesses that developed in response to the Freund's complete adjuvant, or an undetected infectious process. Freund's complete adjuvant stimulates T lymphocytes [74], therefore suppression of cellular immunity may have been masked by the strong inflammatory response to the adjuvant. The DTH, PHA, and SRBC tests all involve T cell responses [33,64,73,111]. The microbicidal assay does not involve the cellular immune system [65,66], and is hypothesized to be less affected by the adjuvant response. The decline in percent killing of *E. coli* and *C. albicans* surrounding oral exposure may be an indication of immune suppression due to imidacloprid, however this cannot be confirmed as no significant differences were detected between treatment groups. The increase in TS over time within all treatment groups may be a reflection of the inflammatory response to the adjuvant, or related to increasing maturity [112,113].

Possible explanations for the lack of observed immunotoxicity in the present study despite documentation in the literature include differences in dose, chemical, exposure method, exposure duration, and immune function assay methodology. Red-legged partridges exposed to an estimated 53 mg/kg/d of imidacloprid via wheat seeds treated with twice the recommended application rate as the only source of food for 10 d demonstrated a decreased PHA response in males, but there was no effect on females and

no effect on SRBC agglutination titers [29]. A second study in red-legged partridges exposed birds to wheat seed treated with 20% or 100% of the recommended application of imidacloprid in 2 separate exposure periods totaling 35 d, and a decreased DTH response was seen only in the offspring of the exposed birds, no immunotoxicity was detected in the exposed adults [28]. These studies used higher doses, longer durations of exposure, and a different exposure method than the present study. Additionally, both of the studies in red-legged partridges detected decreases in cellular immunity, which may have been masked by the robust response to the Freund's complete adjuvant in the present study. Decreased humoral and cellular immunity was observed in domestic chickens after exposure to sublethal doses of thiamethoxam, a different neonicotinoid than the present study [43]. Exposure of 1 to 4 wk old domestic chickens to imidacloprid at 0.05% of the LD_{50} (0.05 mg/kg/day) for 37 d caused decreases in cellular and humoral immunity measured by the antibody response to Newcastle disease vaccine, serum total immunoglobulins, contact hypersensitivity to dinotirochlorobenzene (DNCB), and histopathology of the spleen and bursa of Fabricius [51]. While the imidacloprid dose and species were comparable, birds were younger and exposed for a longer duration of time than the present study, and different immune function assays were used. While immunotoxicity was not detected in domestic chickens using the immune function assays at the doses, dosing interval, and exposure method in the present study, the potential for immunotoxicity in gallinaceous birds due to oral imidacloprid exposure cannot be ruled out based on the data in the peer-reviewed literature. However, based on the findings in the present study, neurotoxicity appears to be a more sensitive endpoint than immunotoxicity to detect sublethal effects from oral imidacloprid exposure.

Future Directions

The United States Environmental Protection Agency currently uses lethal doses for environmental risk assessments [17,19]. The neurobehavioral effects observed in the present study occurred at much lower doses than the LD_{50} and were severe enough to potentially impair survival. Generally only one or 2 species are evaluated for risk assessments, typically mallard ducks (*Anas platyrhynchos*) and northern bobwhite quail (*Colinus virginianus*) [17,19]. Substantial variability exists in the reported LD_{50} of imidacloprid for various species, therefore the observed effect levels are also likely different between species [3,17,19]. In addition to the physiologic differences between species that may translate to variable risk, natural history of each species may also change risk. Further studies are needed to determine the effect levels of various neonicotinoids in other avian orders. The effect doses determined in the present study may be used in environmental risk assessments to incorporate sublethal effects that may impair fitness and survival of wild gallinaceous birds.

Limitations

Potential limitations of the present study include the route of imidacloprid exposure and the use of a model species. Granivorous birds have an extension of their esophagus called the crop which allows for food storage and gradual passage into the ventriculus for digestion [114]. When treated seed is held in the crop and gradually passed to the ventriculus, the time to onset of neurobehavioral signs is likely different than gavage directly into the ventriculus. However, when granivorous birds ingest food

with an empty gastrointestinal tract, the food bypasses the crop and immediately enters the proventriculus and ventriculus [114]. In these cases, the onset of neurobehavioral signs may be similar to what was observed in the present study. Domestic chickens were used as a model for wild granivorous birds assuming that their feeding habits, behavioral and physiological attributes are sufficiently similar to justify their use. Additionally, given the similarities of the amino acid sequences for the nAChR across the order *Galliformes*, the chicken may be a reliable surrogate species for toxicity in this taxon [115]. The neurologic signs observed in the present study are believed to be caused by imidacloprid and/or imidacloprid metabolites stimulating the avian nAChR, therefore, these neurologic signs are likely to occur in other species with comparable receptors.

Conclusion

Domestic chickens exposed to imidacloprid at field-realistic doses developed temporary neurobehavioral signs in a dose-dependent manner. Toxicity was not cumulative, and the birds completely recovered between exposures, indicating that this study is more accurately viewed as a 7 d repeated acute exposure instead of one subacute exposure. A linear relationship existed between severity and duration of neurobehavioral signs, with higher clinical severity scores associated with a longer duration of neurobehavioral signs. Immunotoxicity was found to be a less sensitive indicator of the sublethal effects of imidacloprid than clinical neurobehavioral abnormalities. The present data provides a systematic evaluation of the dose-dependent neurobehavioral signs due to oral imidacloprid exposure in chickens that can be utilized in ecological risk assessments and model development to further evaluate the risk neonicotinoids may pose to wild birds and guide regulations that would minimize these risks.

Figures and Tables

Table 1: Summary of the nominal mg/kg imidacloprid doses and imidacloprid solution concentrations that were used to orally expose domestic chickens. Solution concentrations were confirmed with liquid chromatography tandem mass spectrometry and the confirmed mg/kg doses are presented.

Nominal doses (mg/kg)	Confirmed doses (mg/kg)	Nominal solution concentrations (mg/mL)	Measured solution concentrations (mg/mL)
0.00	0.00	0.00	0.00
0.04	0.03	0.02	0.02
0.34	0.34	0.15	0.15
3.44	3.42	1.50	1.49
10.41	10.25	5.00	4.93
15.62	15.50	7.50	7.45

Table 2: Classification system for the clinical severity scores used to categorize the severity of neurobehavioral signs observed after oral administration of imidacloprid in domestic chickens. The clinical signs used to define each severity score are listed.

Severity Score	Neurobehavioral Signs
0	– No clinical signs
1 = Mild	– Mildly sedate but remains standing – May lie down briefly with other birds but quickly stands when stimulated – Readily returns to normal behavior with external stimulation
2 = Moderate	– Moderate sedation, appears to be sleeping but with abnormal roosting posture (i.e. head down on the ground instead of tucked behind wing) – Consistently lying down – Fluffed feathers – Eyes partially closed or glassy eyed – May demonstrate ataxia – May have intermittent, generalized muscle tremors – Mildly increased respiratory effort – Clinical signs improve to mild with external stimulation but does not return to normal behavior
3 = Severe	– Clinical signs as noted in moderate but more severe – Severe sedation, minimally response to external stimulation – More consistent whole-body tremors – More severe ataxia – Unable to stand or ambulate on own
4 = Comatose	– Severe sedation, non-responsive to external stimulation – More consistent whole-body tremors – Laterally recumbent, unable to stand – Occasional regurgitation

Table 3: Summary of the mean slope ± standard deviation of the clinical severity scores observed in domestic chickens after oral imidacloprid exposure throughout the 7 d exposure period by treatment group. Only treatment groups that developed neurobehavioral abnormalities are included in the table. A Wilcoxon signed rank test was used to determine if the slope within each treatment group differed from zero; these *p* values are presented in the table.

Imidacloprid dose	Slope in both sexes (points/d)	*p* value	Slope in males (points/d)	*p* value	Slope in females (points/d)	*p* value
3.42 mg/kg	-0.07 ± 0.15	0.05	-0.04 ± 0.11	0.40	-0.10 ± 0.18	0.09
10.25 mg/kg	-0.01 ± 0.08	0.80	-0.01 ± 0.07	1.0	-0.01 ± 0.09	0.94
15.50 mg/kg	-0.10 ± 0.10	0.002 ^	-0.05 ± 0.08	0.14	-0.15 ± 0.10	0.01 ^

^ Indicates a statistically significant *p* value

Table 4: Summary of the mean slope ± standard deviation of the duration of neurobehavioral signs observed in domestic chickens after oral imidacloprid exposure throughout the 7 d exposure period by treatment group. Only treatment groups that developed neurobehavioral abnormalities are included in the table. A Wilcoxon signed rank test was used to determine if the slope within each treatment group differed from zero; these *p* values are presented in the table.

Imidacloprid dose	Slope in both sexes (min/d)	*p* value	Slope in males (min/d)	*p* value	Slope in females (min/d)	*p* value
3.42 mg/kg	-0.42 ± 4.69	0.22	-0.30 ± 5.05	0.40	-0.54 ± 4.57	0.40
10.25 mg/kg	1.46 ± 12.67	0.65	-1.11 ± 12.90	0.70	4.03 ± 12.54	0.32
15.50 mg/kg	-6.55 ± 9.51	0.005 ^	-2.33 ± 10.09	0.32	-10.76 ± 7.05	0.004 ^

^ Indicates a statistically significant *p* value

Table 5: Mean hematology values ± standard deviation from domestic chickens in each imidacloprid exposure group (the mg/kg dosing refers to oral imidacloprid exposure) on each sampling date.

Packed cell volume (PCV) %					
mg/kg	Day 0	Day 3	Day 7	Day 14	Day 21
0	28.70 ± 1.49	28.55 ± 2.11	28.85 ± 1.09	30.10 ± 1.83	29.45 ± 2.37
0.03	28.75 ± 1.37	28.20 ± 1.74	27.60 ± 2.01	29.60 ± 1.54	28.20 ± 1.20
0.34	27.80 ± 2.35	28.10 ± 1.68	27.50 ± 2.12	29.95 ± 2.58	28.60 ± 1.67
3.42	28.16 ± 2.83	28.50 ± 1.32	27.25 ± 2.00	29.40 ± 2.01	29.20 ± 1.40
10.25	29.25 ± 1.94	29.40 ± 1.67	28.65 ± 3.82	29.00 ± 1. 52	28.45 ± 2.04
15.50	28.55 ± 1.50	28.75 ± 1.62	28.20 ± 1.64	29.65 ± 1.69	28.80 ± 1.99
Total Solids (TS) g/dL					
mg/kg	Day 0	Day 3	Day 7	Day 14	Day 21
0	3.14 ± 0.38	3.22 ± 0.55	3.32 ± 0.39	3.12 ± 0.49	3.65 ± 0.43
0.03	3.21 ± 0.46	3.25 ± 0.48	3.36 ± 0.40	3.24 ± 0.50	3.72 ± 0.43
0.34	3.01 ± 0.62	3.42 ± 0.47	3.45 ± 0.58	3.47 ± 0.61	3.64 ± 0.46
3.42	3.07 ± 0.51	3.33 ± 0.44	3.43 ± 0.44	3.32 ± 0.46	3.48 ± 0.46
10.25	3.21 ± 0.39	3.37 ± 0.56	3.60 ± 0.55	3.28 ± 0.53	3.78 ± 0.60
15.50	2.73 ± 0.36 *	3.10 ± 0.26	3.35 ± 0.27	3.18 ± 0.22	3.47 ± 0.46
Total White Blood Cell (WBC) Count x 10^3 cells/μL ^					
mg/kg	Day 0	Day 3	Day 7	Day 14	Day 21
0	32.98 ± 5.14	31.12 ± 4.98	34.61 ± 6.22	32.54 ± 5.82	36.31 ± 8.33
0.03	29.85 ± 6.20	31.51 ± 6.81	34.29 ± 5.56	31.63 ± 7.31	41.70 ± 10.68 [a]
0.34	32.48 ± 7.20	35.50 ± 8.81	36.74 ± 6.56	37.80 ± 9.54	42.09 ± 15.29 [a]
3.42	28.95 ± 7.52	34.08 ± 8.97	35.13 ± 6.37	36.80 ± 9.42	36.40 ± 8.30 [a]
10.25	31.04 ± 7.57	31.50 ± 6.44	37.53 ± 8.68	36.35 ± 11.76	43.19 ± 12.17 [a]
15.50	31.89 ± 6.42	31.76 ± 6.26	38.78 ± 7.68	36.43 ± 4.16	38.71 ± 9.61 [a]
Heterophil:Lymphocyte (H/L) Ratio					
mg/kg	Day 0	Day 3	Day 7	Day 14	Day 21
0	0.16 ± 0.09	0.36 ± 0.17	0.28 ± 0.19	0.25 ± 0.13	0.52 ± 0.43
0.03	0.25 ± 0.28	0.30 ± 0.19	0.32 ± 0.15	0.34 ± 0.34	0.63 ± 0.50 [b]
0.34	0.22 ± 0.15	0.35 ± 0.18	0.35 ± 0.23	0.44 ± 0.40	0.62 ± 0.53 [b]
3.42	0.19 ± 0.14	0.38 ± 0.28	0.32 ± 0.17	0.41 ± 0.32	0.47 ± 0.28 [b]
10.25	0.18 ± 0.12	0.30 ± 0.15	0.39 ± 0.20	0.33 ± 0.33	0.69 ± 0.66 [b]
15.50	0.20 ± 0.16	0.29 ± 0.18	0.37 ± 0.16	0.19 ± 0.08	0.31 ± 0.20

^ Total WBC counts are not corrected by PCV

* Significantly less than 0.03 and 10.25 mg/kg

[a] Significantly higher total WBC count on day 21 compared to day 0 within the same treatment group

[b] Significantly higher H/L ratio on day 21 compared to day 0 within the same treatment group

Table 6: Summary table of the immunologic and necropsy results. Mean values are presented for each treatment group ± standard deviation and did not differ between groups. The phytohemagglutinin-A (PHA) response is the post PHA injection skin measurement subtracted from the pre PHA injection skin measurement. The delayed-type hypersensitivity (DTH) response is expressed as the following ratio: (post purified protein derivative (PPD) injection measurement/pre-PPD injection measurement) – (post saline injection measurement/pre-saline injection measurement). The sheep red blood cell (SRBC) agglutination titers are expressed as the $\log_2$ of the reciprocal titer. The hemolysis results are expressed as the plasma dilution required to produce 50% lysis of the SRBCs (CH_{50}). Organ weights at necropsy are expressed as a percentage of bird body weight.

	0 mg/kg	0.03 mg/kg	0.34 mg/kg	3.42 mg/kg	10.25 mg/kg	15.50 mg/kg
PHA response (mm)	0.61 ± 0.16	0.61 ± 0.17	0.68 ± 0.30	0.57 ± 0.14	0.68 ± 0.27	0.62 ± 0.21
DTH response (mm)	0.21 ± 0.14	0.25 ± 0.15	0.23 ± 0.17	0.19 ±0.14	0.22 ± 0.14	0.24 ± 0.15
SRBC agglutination titer	4.10 ± 1.67	4.75 ± 2.40	5.27 ± 2.26	3.61 ± 1.26	4.56 ± 1.54	4.37 ± 1.33
CH_{50} hemolysis titer	11.41 ± 6.38	15.14 ± 12.79	14.21 ± 8.36	10.46 ± 6.88	13.68 ± 8.03	11.80 ± 5.88
Spleen (%)	0.28 ± 0.07	0.30 ± 0.06	0.29 ± 0.06	0.28 ± 0.06	0.30 ± 0.05	0.27 ± 0.06
Thymus (%)	0.48 ± 0.19	0.47 ± 0.21	0.46 ± 0.18	0.47 ± 0.18	0.47 ± 0.24	0.54 ± 0.16
Bursa of Fabricius (%)	0.36 ± 0.17	0.36 ± 0.10	0.31 ± 0.09	0.34 ± 0.15	0.36 ± 0.16	0.38 ± 0.12
Liver (%)	4.12 ± 0.47	4.19 ± 0.37	4.17 ± 0.43	4.16 ± 0.37	4.41 ± 0.62	3.98 ± 0.33

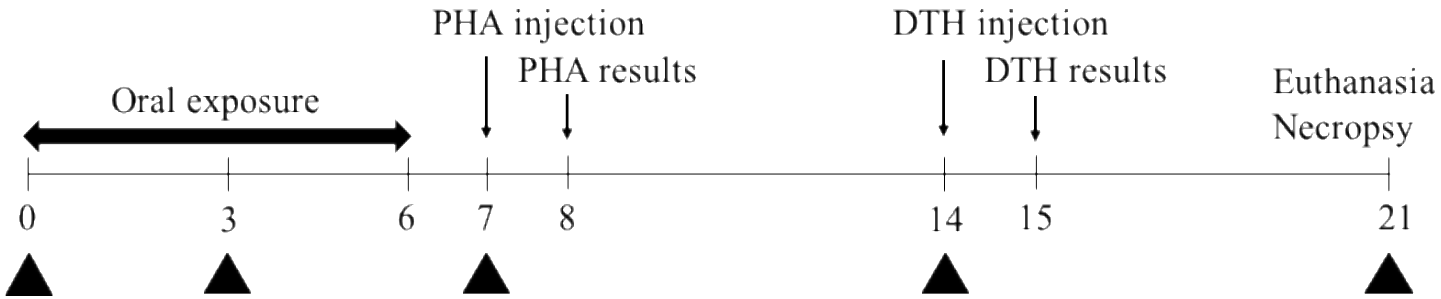


Figure 1: Graphical depiction of the study timeline including key diagnostic tests. The numbers on the horizontal timeline indicate the study day. The black triangles indicate blood collection for a complete blood cell analysis and microbiocidal assay. PHA refers to the phytohemagglutinin-A response test, DTH refers to the delayed type hypersensitivity test.

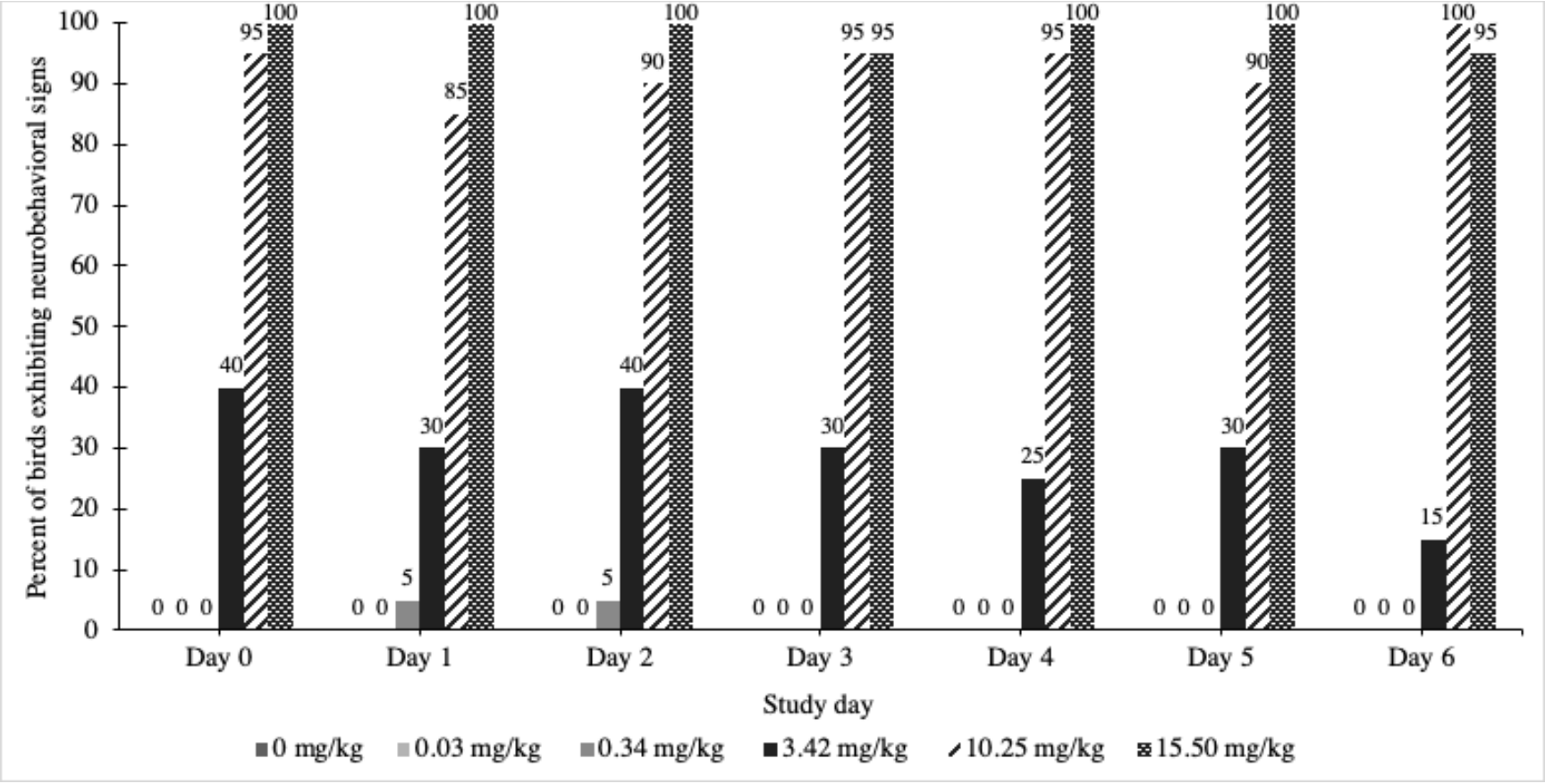


Figure 2: Percent of domestic chickens within each oral imidacloprid exposure group that developed any degree of neurobehavioral abnormalities on each day of oral gavage.

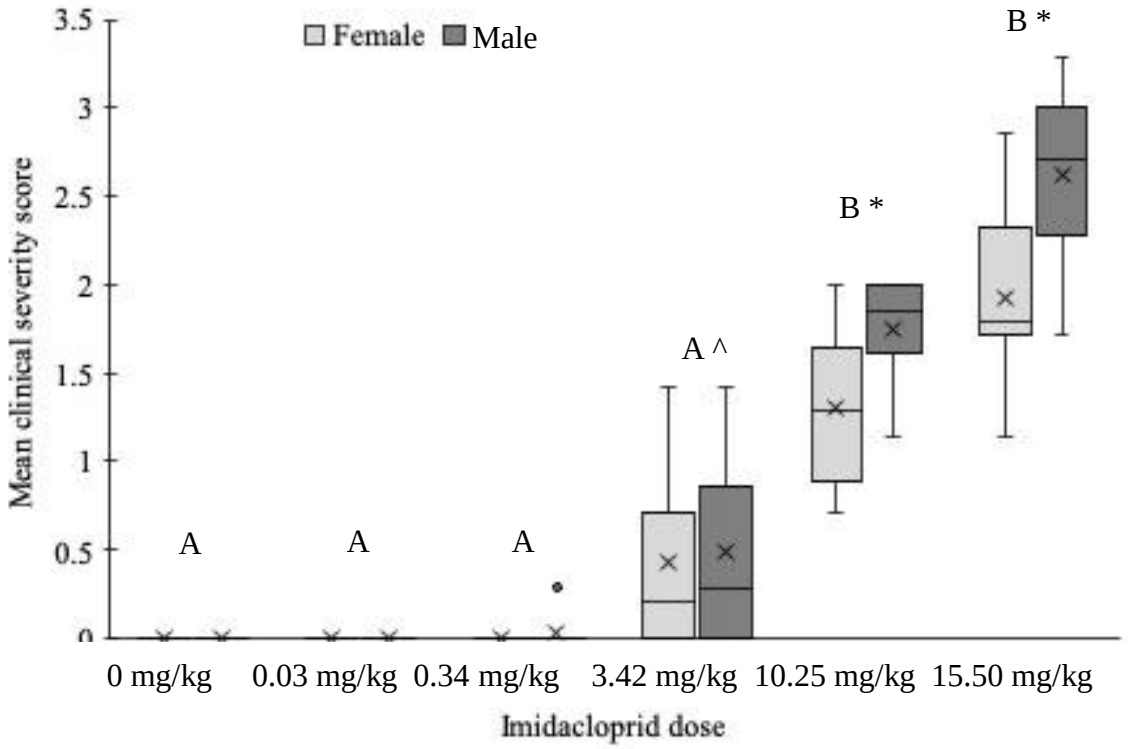


Figure 3: Box and whisker plot depicting the mean clinical severity score in domestic chickens after oral imidacloprid exposure by treatment group and sex. The bottom and top of the box represent the first and third quartiles respectively, the horizontal line within the box indicates the median, the "X" within the box indicates the mean, the ends of the whiskers mark one standard deviation above and below the mean, and the circle above the 0.34 mg/kg group represents the single outlier.

Alphabetical letters above the treatment groups indicate statistically significant differences between treatment groups, where groups with different letters are statistically different from each other.

* Indicates statistically significant differences between sexes within the same treatment group

^ Indicates marginally higher mean clinical severity score compared to 0 mg/kg

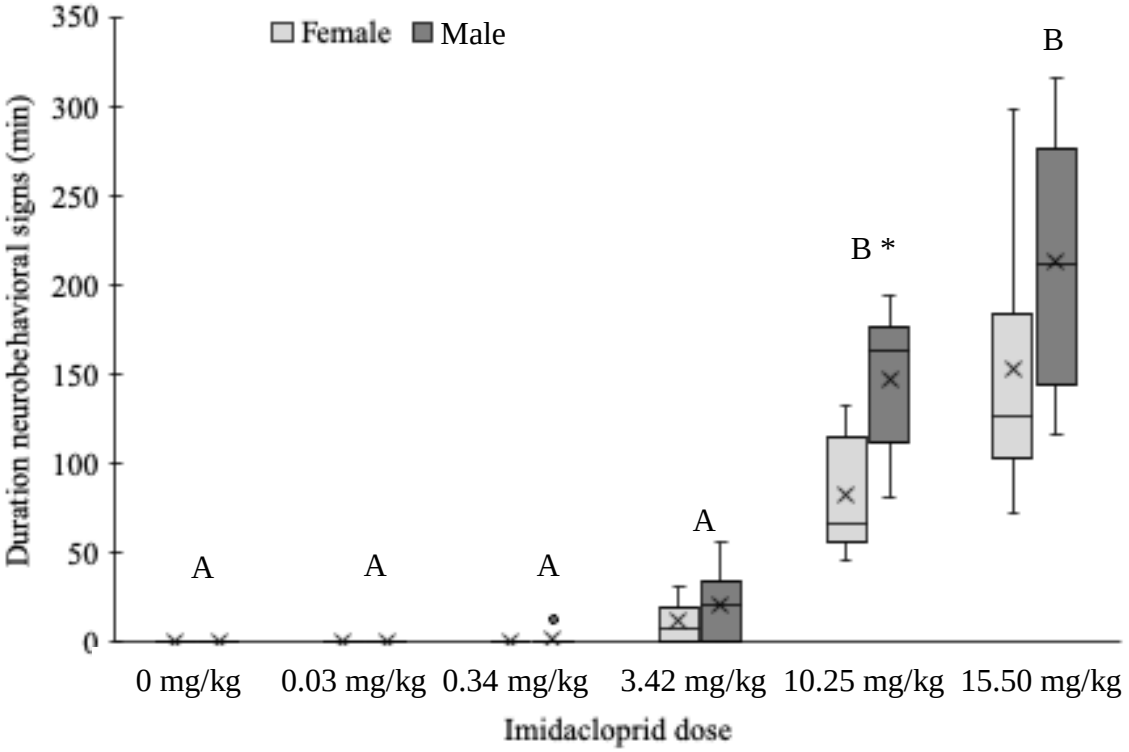


Figure 4: Box and whisker plot depicting the mean duration of neurobehavioral signs in domestic chickens after oral imidacloprid exposure by treatment group and sex. The bottom and top of the box represent the first and third quartiles respectively, the horizontal line within the box indicates the median, the "X" within the box indicates the mean, the ends of the whiskers mark one standard deviation above and below the mean, and the circle above the 0.34 mg/kg group represents the single outlier.

Alphabetical letters above the treatment groups indicate statistically significant differences between treatment groups, where groups with different letters are statistically different from each other.

* Indicates statistically significant difference between sexes within the same treatment group

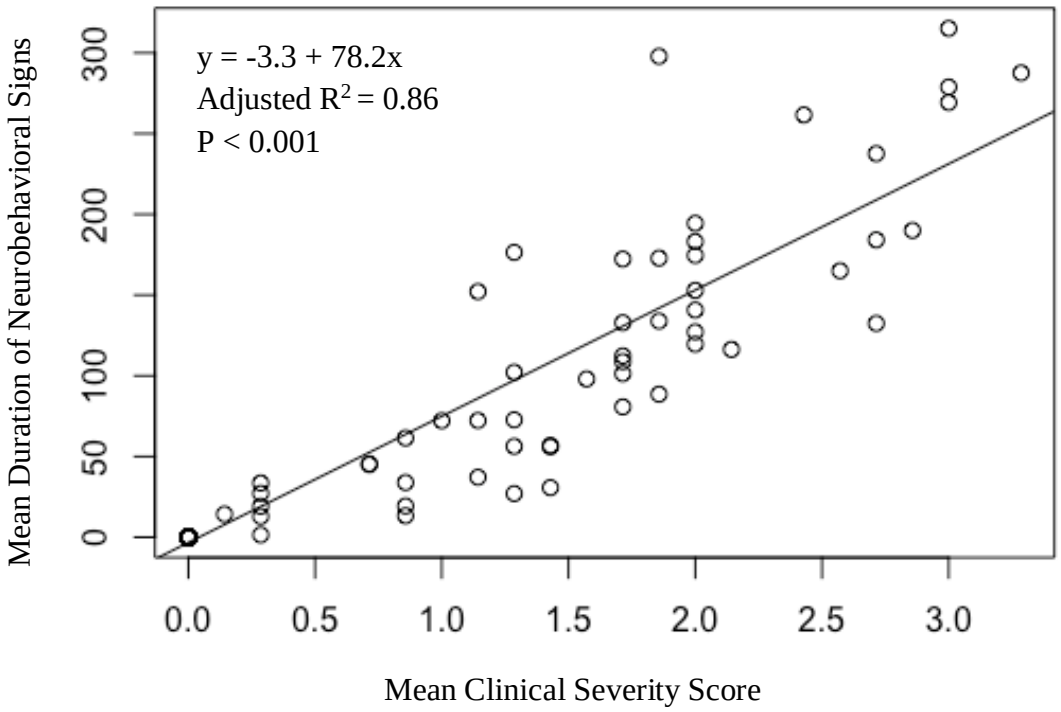


Figure 5: Linear regression plot using the mean clinical severity score as the predictor and mean duration of neurobehavioral signs for each chicken after oral imidacloprid exposure as the outcome variable.

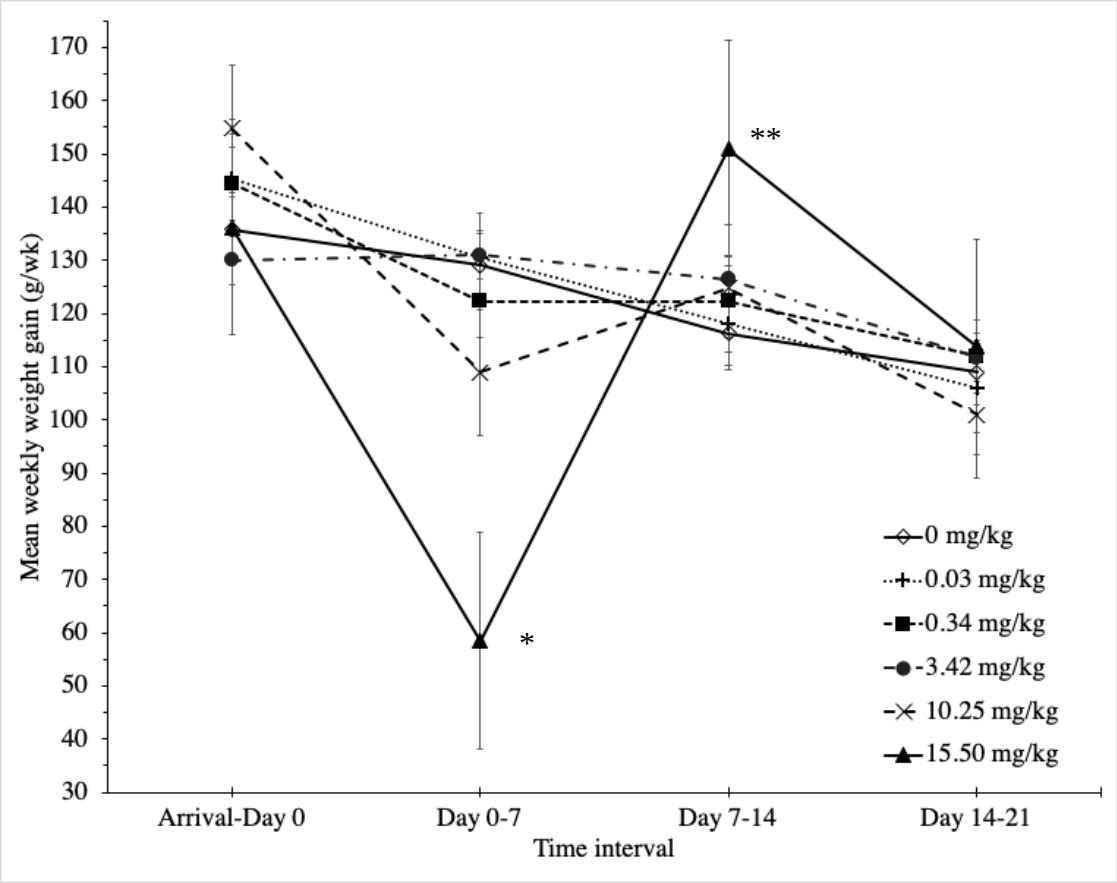


Figure 6: Mean weekly weight gain (± standard error) of domestic chickens in each imidacloprid exposure group in grams per week (g/wk); statistically significant differences between treatment groups each week are noted with asterisks.

* Significantly decreased weight gain in the 15.50 mg/kg group compared to all other treatment groups

** Significantly increased weight gain in the 15.50 mg/kg group compared to all other treatment groups

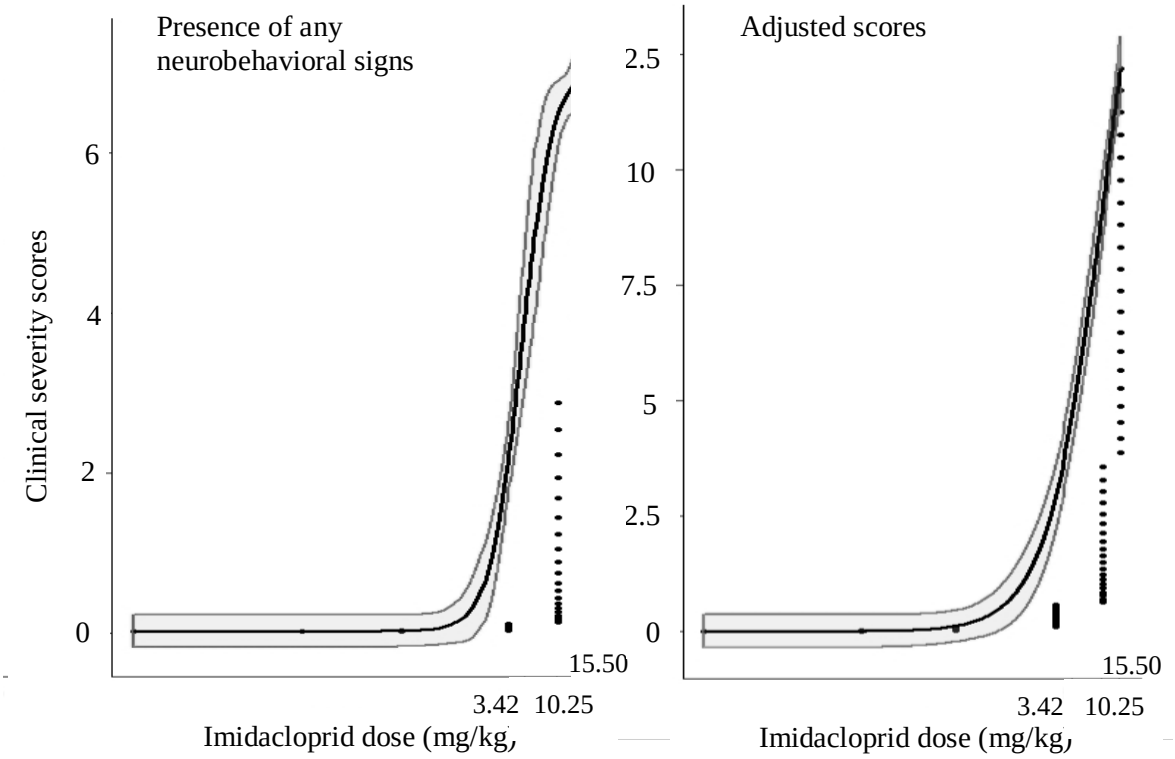
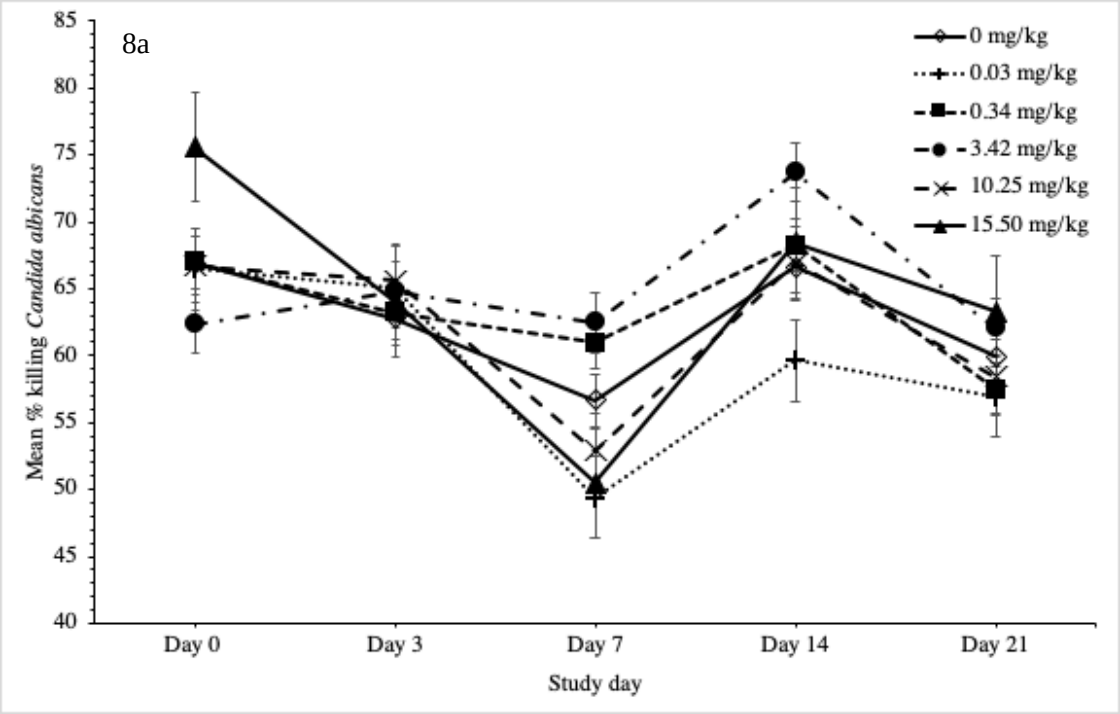
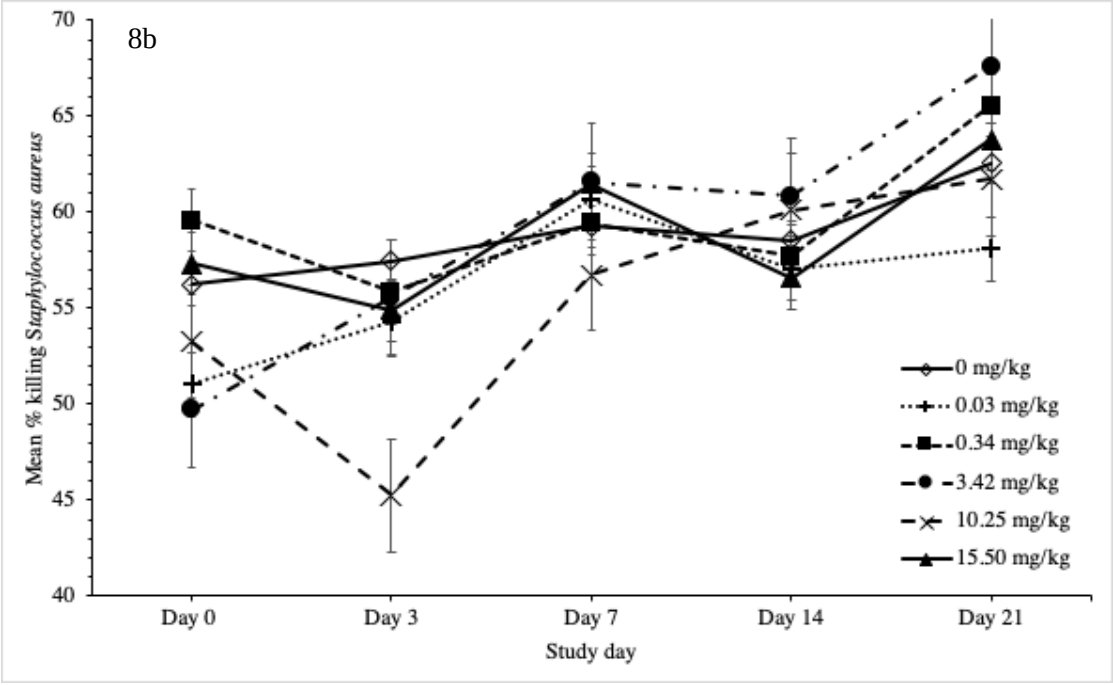


Figure 7: Effect dose (ED) dose-response curves in domestic chickens using the presence of any neurobehavioral signs and the adjusted clinical severity scores after oral imidacloprid exposure.


8a
Mean % killing Candida albicans
85
80
75
70
65
60
55
50
45
40
Day 0
Day 3
Day 7
Day 14
Day 21
Study day
0 mg/kg
0.03 mg/kg
0.34 mg/kg
3.42 mg/kg
10.25 mg/kg
15.50 mg/kg


8b
Mean % killing Staphylococcus aureus
70
65
60
55
50
45
40
Day 0
Day 3
Day 7
Day 14
Day 21
Study day
0 mg/kg
0.03 mg/kg
0.34 mg/kg
3.42 mg/kg
10.25 mg/kg
15.50 mg/kg

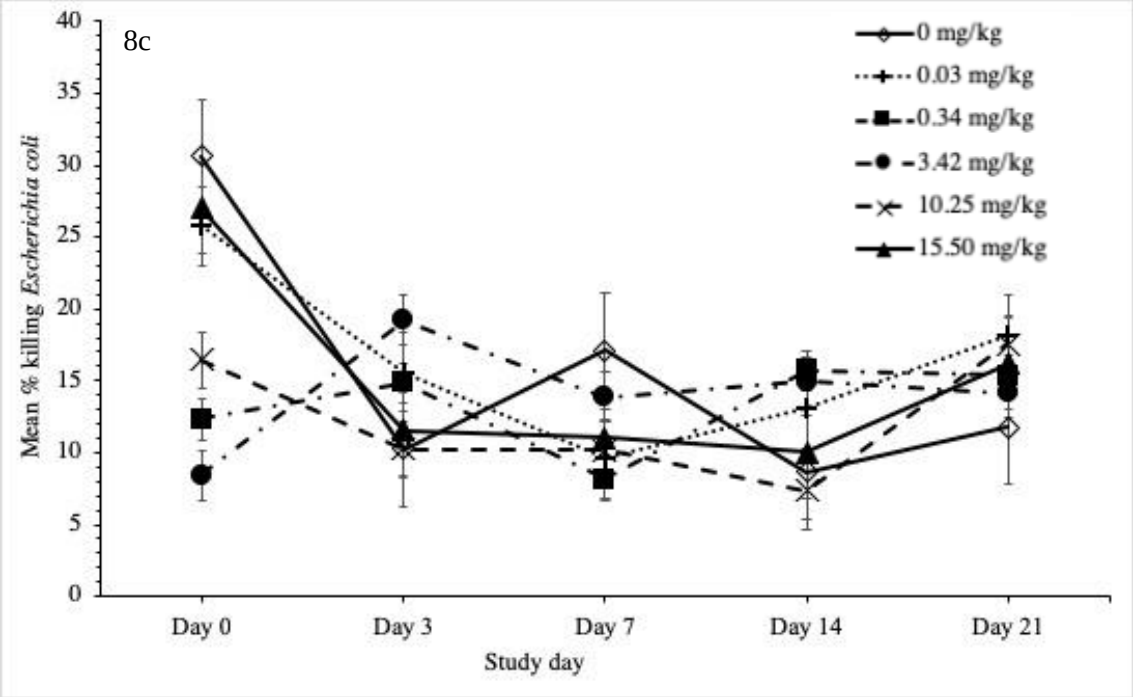


Figure 8: Mean percent killing of each microorganism (± standard error) in domestic chickens by imidacloprid exposure group expressed as percent killing for each sampling date. *Candida albicans* is in figure 8a, *Staphylococcus aureus* is in figure 8b, and *Escherichia coli* is in figure 8c. Statistically significant differences between treatment groups are notated with asterisks.

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